

THE RESPIRATORY ENZYMES OF DIAPAUSING SILKWORM PUPAE: A NEW INTERPRETATION OF CARBON MONOXIDE-INSENSITIVE RESPIRATION ¹

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The respiration of most organisms is inhibited in large measure by carbon monoxide. This indicates that cytochrome oxidase is the main terminal enzyme in electron transfer (Warburg, 1949; Keilin and Slater, 1953). But as is well known to students of insect physiology, the respiration of many diapausing insects is remarkably insensitive to cyanide, carbon monoxide, and other inhibitors of cytochrome oxidase. The significance of this insensitivity was recently discussed by Harvey and Williams (1958a, 1958b) as a result of studies on the heart of diapausing pupae of the *Cecropia* and *Polyphemus* silkworms. Quite independently we have carried out a detailed study of another aspect of this phenomenon (Kurland, 1957). Our attention has centered, not on a single organ such as the heart, but on the respiration of the whole insect. These two investigations prove complementary in the analysis of the problem as a whole.

Carbon monoxide-insensitive respiration in insects was first detected by Bodine and Boell (1934a, 1934b) who reported that the oxygen consumption of diapausing eggs of the grasshopper *Melanoplus* was not inhibited by carbon monoxide. Later, Allen (1940) showed that the cytochrome *c* oxidase activity of these diapausing eggs was high, despite the insensitivity of their respiration to both carbon monoxide and cyanide. He concluded (p. 162) that the "rates of oxygen consumption of pre-diapause, diapause, and very early post-diapause eggs are independent of the relative amounts of cytochrome oxidase." An important clue to the reconciliation of the CO-insensitivity of diapausing *Melanoplus* eggs with the simultaneous presence of cytochrome oxidase was provided by Bodine and Boell (1936, 1938). They discovered that 2,4-dinitrophenol (DNP) increased the respiration of diapausing eggs and that this increased respiration was inhibited by carbon monoxide and cyanide. Unfortunately, the significance of this observation could not be fully comprehended because the mechanism of DNP action was not explained until a decade later (Loomis and Lipmann, 1948).

As the result of an intensive investigation of the CO-insensitivity of pupal respiration in the giant silkworm *Hyalophora cecropia*, Schneiderman and Williams (1952, 1954a, 1954b) concluded that the cytochrome *c* oxidase system was not functioning in most tissues of the diapausing pupa, although it functioned at all other

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stages in the life history. Their arguments have recently been summarized (Lees, 1956; Schneiderman, 1957). They suggested that pupal respiration was mediated by an autoxidizable flavoprotein or a heme-containing enzyme insensitive to carbon monoxide. This explanation was supported by the observations of Shappirio and Williams (1953), Shappirio (1954), and Pappenheimer and Williams (1953, 1954) who reported the existence of a new autoxidizable cytochrome component (e or b_5) in *Cecropia* pupae. Also, Chefurka and Williams (1952) reported an increased amount of flavoprotein in pupal tissues. However, there was no evidence to indicate that the new cytochrome or the flavoprotein functioned as a terminal oxidase in the pupal respiratory chain.

The experiments reported here continue these earlier studies and were prompted, in part, by recent advances in our understanding of electron transfer in the cytochrome system (*cf.* review by Chance and Williams, 1956). We examined the effects on respiration of two inhibitors of cytochrome oxidase, carbon monoxide and sodium azide, and also of antimycin A, a potent inhibitor of the DPNH-cytochrome c reductase system. In addition we studied the effects of 2,4-dinitrophenol which dissociates phosphorylation from oxidation. It was hoped that a study of the action of these rather specific inhibitors on pupae in various metabolic states might permit a decisive definition of the terminal oxidase of diapausing pupae. This objective has been achieved. The results of the present study, coupled with the recent findings of Harvey and Williams (1958b), have enabled us to identify this oxidase as cytochrome oxidase and thus contradict earlier conclusions. The experiments also reveal some new biochemical peculiarities of the diapause condition.

MATERIALS AND METHODS

1. *Experimental animals*

Diapausing pupae of *Hyalophora* (*Platysamia*) *cecropia* (4 to 6 gm.), *Callosamia* *promethea* ($1\frac{1}{2}$ to $2\frac{1}{2}$ gm.), *Samia* *cynthia* ($1\frac{1}{2}$ to $3\frac{1}{2}$ gm.) and *Antheraea* (*Teia*) *polyphemus* (4 to 6 gm.) were used as experimental animals. In our experience diapausing pupae of these four species of closely related saturniid moths behave in virtually identical fashion in respiration experiments and hence we have used them interchangeably. The animals were reared under field conditions or collected in nature and were stored at 25° C. for a minimum of four months before use in experiments. One group of *Cynthia* and *Promethea* pupae was maintained at 5° C. for several months and then returned to 25° C., whereupon their brains were removed. This brain removal put the pupae in a state of permanent diapause (Williams, 1946) and, after three months at 25° C., these animals behaved in experiments like normal unchilled diapausing pupae. Only pupae displaying a relatively constant respiratory rate over a period of at least six hours were used in experiments. Also, since it was shown by Schneiderman and Williams (1954a) that cellular respiration of the pupal abdominal muscles is mediated by cytochrome oxidase, pupae showing excessive muscular activity were excluded.

2. *Measurement of respiration*

The present investigation is based on more than 2000 respiratory measurements performed on about 500 pupae. Rates of oxygen consumption were determined

manometrically according to techniques described previously (Schneiderman and Williams, 1953a). Measurements were carried out in 50-cc. vessels equipped with venting plugs and adapters for use with standard Warburg manometers.

3. *Gas mixtures*

In some experiments, pupae were exposed to various gas mixtures while enclosed in the Warburg vessels. Commercial gases were purified and gas mixtures prepared and analyzed by methods described previously (Schneiderman and Williams, 1954a). All of the experiments were performed at atmospheric pressure. The vessels were periodically re-flushed during the course of the experiments, a maneuver which prevented any significant reduction of oxygen tension within the vessels. Appropriate control vessels were run in all experiments to take into account the manometric effect of reactions between carbon monoxide and the alkali.

4. *Reagents*

Sodium azide and 2,4-dinitrophenol were reagent grade. Crystalline antimycin A, obtained from the Wisconsin Alumni Research Foundation, was dissolved in aqueous ethanol. The final dilutions of antimycin A injected into the pupae were uniformly in 1% ethanol solutions.

Previous to injection, the pupae were anesthetized with carbon dioxide. In our experience the respiration of diapausing pupae is not significantly affected by thirty minutes of carbon dioxide anesthesia. Approximately 0.1 cc. of solution was injected *via* a 26-gauge needle into each pupa. The final concentrations within the animal were calculated on the basis of a pupal water content of 70 per cent. Respiration was measured for a minimum of three hours after injection.

5. *Interpretation of inhibitor experiments*

The act of piercing merely the skin of a diapausing pupa with a fine hypodermic needle causes a prompt stimulation of respiration for several hours. This is followed by a subsequent slow rise in respiration—injury respiration (Schneiderman and Williams, 1953a, 1953b). Hence in interpreting inhibitor experiments, it is necessary to separate the effects of injury from those of the chemical injected (Schneiderman and Williams, 1954a). This can best be accomplished by comparing experimental pupae with control pupae injected with a corresponding volume of the solvent used, *e.g.*, 1% ethyl alcohol, distilled water, etc. Furthermore, it is simplest to make comparisons soon after injection, before injury respiration increases to high levels and possibly before the injected chemical is detoxified or otherwise metabolized. In most of the inhibitor experiments to be reported, the pupae had a very low basal metabolic rate and simple injection commonly doubled their oxygen consumption.

6. *Injury*

Pupae were anesthetized with carbon dioxide. Injuries were made either by removing a rectangle of pupal cuticle and underlying hypodermis from the face or by excising the pupal legs. The wounds were then covered with plastic windows sealed in place with paraffin. A few crystals of streptomycin sulfate and phenyl-

thiourea (a 1:1 mixture) were placed in the wounds to prevent infection and to prevent darkening of the blood by tyrosinase (Williams, 1952; Schneiderman and Williams, 1953a).

EXPERIMENTAL RESULTS

1. *Diapause respiration*

A. *The development of CO-insensitive respiration after pupation*

The effects of carbon monoxide on the respiration of newly pupated *Cecropia* silkworms were observed at intervals over a ten-day period. The pupae were exposed first to a nitrogen-oxygen mixture and then to a carbon monoxide-oxygen mixture. The results, as well as details of the procedure, are recorded in Table I. As the pupae aged they exhibited a gradual decrease in their respiratory rate which

TABLE I

*The development of CO-insensitive respiration in four newly molted Cecropia pupae**

Age after pupation (hrs.)	Respiration in nitrogen mixture (mm. ³ /gm./hr.)	% insensitive respiration
5	34	59
29	26	49
197	7	92
6	37	51
30	28	69
198	7	86
19	26	47
43	25	74
211	7	80
19	26	57
43	23	55
211	10	80

* Pupae were exposed for three hours to an atmosphere of 6 per cent oxygen and 94 per cent nitrogen, and then for three hours to an atmosphere of 6 per cent oxygen and 94 per cent carbon monoxide. To calculate per cent insensitive respiration, oxygen consumption in the carbon monoxide mixture was compared to oxygen consumption in the nitrogen mixture.

was accompanied by a marked decrease in the fraction of respiration sensitive to carbon monoxide. Thus while immediately after pupation half their respiration was inhibited by carbon monoxide, 200 hours later less than 20 per cent was CO-sensitive.

B. *The CO-insensitivity of respiration of diapausing pupae*

Figure 1 records the per cent of CO-insensitive respiration for a large number of pupae with different basal rates of oxygen consumption. The data show that as oxygen consumption increases, respiration becomes increasingly sensitive to carbon monoxide. However, it is of special interest that even when carbon monoxide inhibited the respiration of diapausing pupae it rarely inhibited more than 20 per cent of their total respiration, and for pupae whose basal respiration was between 15 and 20 mm.³/gm. live wt./hr., the respiration appeared to be unaffected by carbon monoxide. It is also noteworthy that carbon monoxide appeared to stimulate

the respiration or at least the *gas uptake* of pupae whose basal oxygen consumption was less than 15 mm.³/gm. live wt./hr. We have duplicated these results in numerous experiments with *Cynthia*, *Polyphemus* and *Promethea* pupae. In all cases the apparent stimulation was greatest for pupae with low basal respiratory rates and possible explanations for this phenomenon will be offered in the Discussion. But

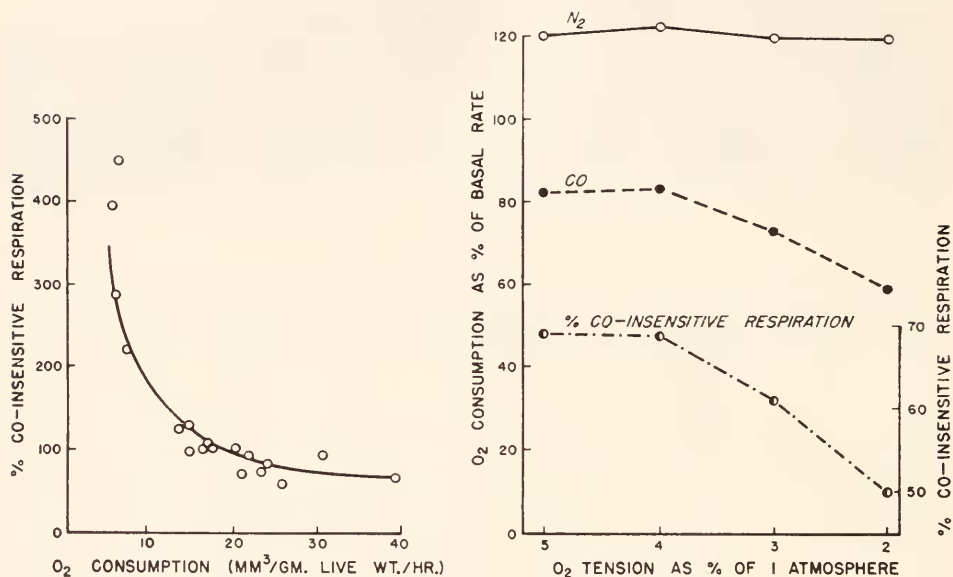


FIGURE 1 (left). The CO-insensitivity of pupal respiration as a function of basal O₂ consumption. CO/O₂ ratio = 19:1. The oxygen tension was 5% in both CO and N₂ mixtures. The gas exchange of 18 diapausing *Cynthia* pupae was measured first in the N₂ mixture and then in the CO mixture. The per cent of CO-insensitive respiration is plotted against the respiration in the N₂ mixture.

FIGURE 2 (right). The CO-sensitivity of pupal respiration at reduced O₂ tensions. The average O₂ consumption of four brainless *Promethea* pupae whose average O₂ consumption in air was 33 mm.³/gm. live wt./hr. is recorded at each successive O₂ tension in the O₂-N₂ mixtures. Similarly the average O₂ consumption of five other pupae, whose average O₂ consumption in air was 27 mm.³/gm. live wt./hr., is recorded at each O₂ tension in the O₂-CO-N₂ mixtures. The rate of respiration in each gas mixture is expressed as per cent of basal rate in air. The CO/O₂ ratio was kept constant at 19:1 by adding appropriate amounts of N₂ to the O₂-CO mixture. The right-hand vertical axis records the per cent of CO-insensitive respiration. Oxygen consumption decreased at tensions below 2% and this so complicated measurements of CO inhibition that values for O₂ tensions below 2% could not be calculated. The average weight of the pupae was 2 grams.

for the present, suffice it to note that in the presence of carbon monoxide the *gas uptake* of pupae which have low respiratory rates in air is markedly increased and that this fact complicates studies of carbon monoxide inhibition on these animals.

C. The CO-sensitivity of pupal respiration at reduced oxygen tensions

In the following experiment the CO-sensitivity of the respiration of a group of *Promethea* pupae was measured at oxygen tensions ranging from 5 to 2 per cent of an atmosphere. The results summarized in the lower curve in Figure 2 disclose

that at low oxygen tensions pupal respiration becomes sensitive to carbon monoxide. As the figure reveals, pupal respiration was not depressed by low oxygen alone down to 2 per cent. In sharp contrast to this insensitivity of respiration to low oxygen tensions in the nitrogen-oxygen mixtures, the respiration in 19:1 CO/O₂ remained constant only between 5 and 4 per cent oxygen and then progressively decreased as the oxygen tension decreased. In other words, at low oxygen tensions pupal respiration is inhibited by carbon monoxide. These observations suggest that cytochrome oxidase is functioning at all times in the diapausing pupa but at higher oxygen tensions its CO-sensitivity is in some manner masked. A similar experiment performed on *Cynthia* pupae yield substantially the same results.

The cause of the slight stimulation of respiration shown in the figure in nitrogen-oxygen mixtures containing 2 per cent oxygen or more is unknown. In 1 per cent oxygen respiration fell to about 85 per cent of the basal rate. It is significant that these measurements were conducted on *Prometha* pupae less than one half the size of *Cecropia* pupae used by Schneiderman and Williams (1954a) in an experiment appraising the effect of oxygen tension on pupal respiration. They reported that the respiration of *Cecropia* pupae decreased when oxygen tension fell below 5 per cent. These contrasting results are explained by the fact that in the larger *Cecropia* pupae the diffusion distances are greater than in *Prometha* pupae. Therefore, the actual tension of oxygen within the pupal tissues is probably less for large pupae than small ones. As a result, the respiration of large pupae is limited at oxygen tensions which do not affect the respiration of small pupae.

D. The effects of sodium azide and antimycin A on pupal respiration

Three groups of five diapausing *Cynthia* pupae, whose average basal respiration was 15.4 mm.³/gm. live wt./hr., were injected with sodium azide to internal concentrations of 10^{-5} M, 10^{-4} M, and 5×10^{-4} M. The average respiration of these pupae on the day of injection was indistinguishable from the respiration of five control pupae injected with water. Since azide is an extremely soluble small molecule, it is doubtful that impermeability is responsible for this insensitivity. Pupal respiration is thus relatively insensitive to azide as well as to carbon monoxide.

In a similar experiment, the effects of antimycin A on respiration were examined in fifteen *Cynthia* pupae whose average basal respiration was 13.3 mm.³/gm. live wt./hr. A control group of five received a 1 per cent ethyl alcohol solution, a second group received antimycin A to an internal concentration of 10^{-7} M and the third group received antimycin A to a final concentration of 10^{-6} M. Injecting the 1 per cent ethyl alcohol had exactly the same effect as injecting distilled water and promptly doubled the respiration. Compared with the ethyl alcohol control, 10^{-7} antimycin A inhibited respiration about 20 per cent and 10^{-6} M about 30 per cent. Similar concentrations of antimycin A commonly cause much higher inhibitions in other organisms and the respiration of the diapausing pupa may be considered relatively insensitive to this potent inhibitor of the cytochrome *c* reductase system.

2. DNP-stimulated respiration

A. The stimulatory effect of DNP

An important clue to the nature of the oxidative pathways of diapausing pupae was uncovered in 1955 by Harvey and Shappirio (unpublished observations) who

discovered that DNP stimulated the respiration of diapausing *Cecropia* pupae and that this respiration was CO-sensitive. This result, which they generously shared with us, agreed with the earlier observations of Bodine and Boell noted in the Introduction, and suggested to us a number of experiments using DNP.

A series of *Cynthia* pupae were injected with DNP to internal concentrations ranging from $5 \times 10^{-4} M$ to $10^{-5} M$. Figure 3 records the average initial stimula-

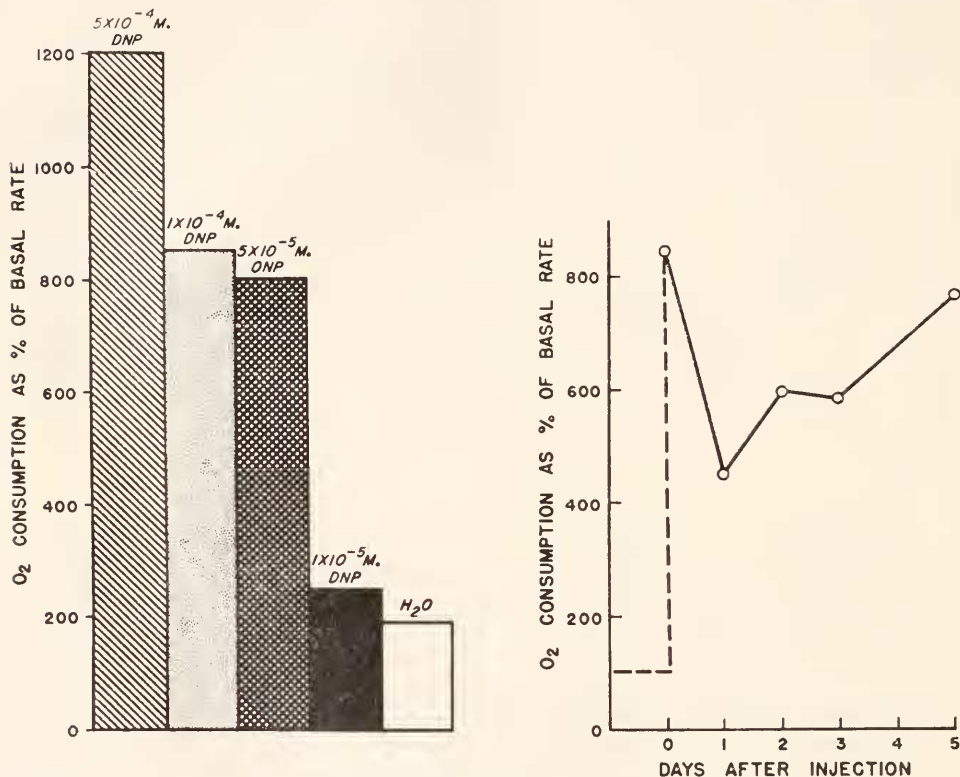


FIGURE 3 (left). The effect of several concentrations of DNP on the respiration of diapausing pupae. Five *Cynthia* pupae received injections of DNP to an internal concentration of $5 \times 10^{-4} M$, four received $10^{-4} M$, four received $5 \times 10^{-5} M$, four received $10^{-5} M$, and a control group of four received distilled water. The average O₂ consumption for the first three hours after injection is recorded.

FIGURE 3 (right). The effect of DNP injection on the O₂ consumption of diapausing pupae. Four *Cynthia* pupae whose average O₂ consumption was 13.0 mm.³/gm. live wt./hr. were injected with DNP to an internal concentration of $1 \times 10^{-4} M$. Respiration was measured for three hours each day over a five-day period. The day of injection is denoted as day "0".

tion of respiration of several concentrations for the first three hours after injection, while Figure 4 records the respiratory behavior of the $10^{-4} M$ group over a five-day period. Dinitrophenol called forth an immediate and spectacular increase in oxygen consumption which averaged 12 times the basal rate in the case of pupae receiving $5 \times 10^{-4} M$. As Figure 4 shows, in the group receiving $10^{-4} M$ the initial acceleration of respiration on the day of injection was followed by a decline on the following

day. This was succeeded by a gradual increase of respiration over a three-day period, to a peak on the fifth day after injection almost as great as the initial peak respiration. The respiration returned to about normal approximately two weeks later. The initial stimulation of respiration is doubtless due to the uncoupling effect of DNP which causes an acceleration of the turnover rate of the components of the respiratory chain (Cross *et al.*, 1949; Chance and Williams, 1956). The secondary effects which develop several days later, appear to be the result of (a) injury-stimulated respiration provoked by injection through the cuticle (see Section 3 below) and

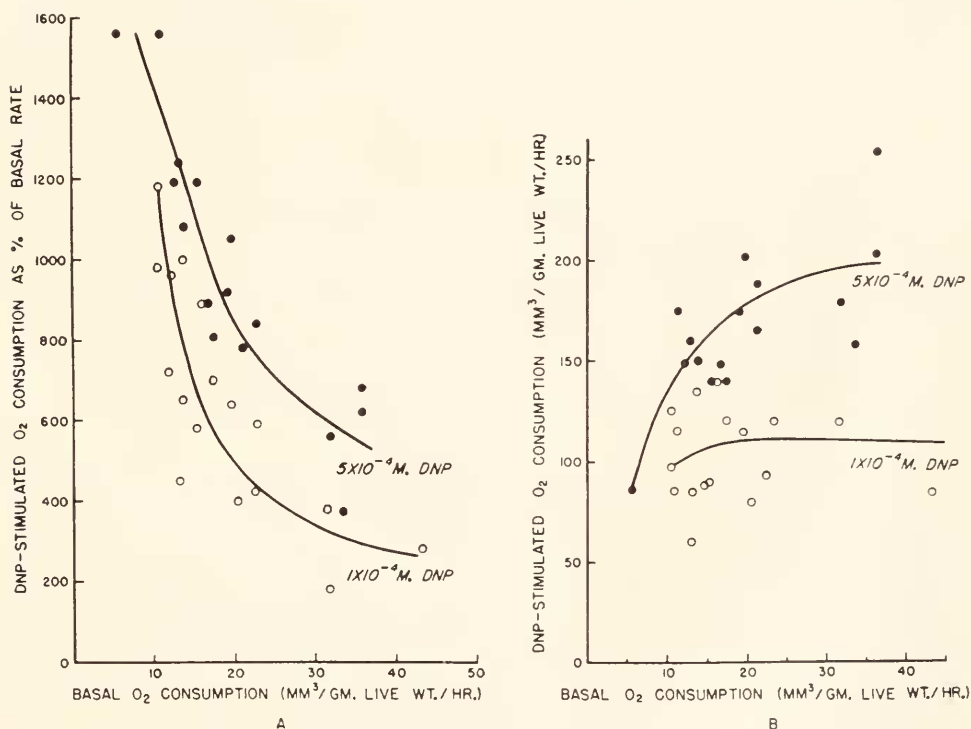


FIGURE 5. The stimulation of O₂ consumption by DNP as a function of basal respiratory rate. (A) The percentage stimulation of O₂ consumption of *Cynthia* pupae after injection of DNP to internal concentrations of $5 \times 10^{-4} M$ and $10^{-4} M$ is plotted as a function of basal respiration. (B) The total DNP-stimulated respiration of the pupae in (A) is plotted as a function of the basal respiration.

(b) the development of an "energy debt" metabolism (analogous to an "oxygen debt repayment" (Kurland *et al.*, 1958)) as the result of prolonged uncoupling of phosphorylation by DNP. Comparable results were obtained with diapausing pupae of *Cecropia*, *Promethea* and *Polyphemus*.

The time course of the respiratory changes recorded in Figure 4 is also typical of pupae receiving $5 \times 10^{-4} M$ DNP but the pattern differed somewhat in pupae that received lower concentrations. Because the initial stimulation of respiration was less, the fall in respiration recorded in Figure 4 was commonly absent. The

5×10^{-4} M concentration is apparently close to the lethal level and occasional individuals died about a week after receiving that amount.

Further analysis of the effects of DNP disclosed that pupae with high initial basal respirations were proportionately less stimulated by DNP than were pupae with low basal metabolic rates. Figure 5A shows that a *Cynthia* pupa with a basal metabolic rate of $5 \text{ mm}^3/\text{gm. live wt./hr.}$ experienced a 16-fold stimulation of respiration after injection of DNP whereas a similar pupa with a basal respiration of $30 \text{ mm}^3/\text{gm. live wt./hr.}$ experienced only a 6-fold stimulation of respiration. Thus, there is a steep decline in the per cent of DNP-stimulated respiration as basal respiration increases. Figure 5B further reveals that DNP-stimulated respiration approaches a limit as the basal respiration approaches $25 \text{ mm}^3/\text{gm. live wt./hr.}$ The significance of this limit will be considered in the Discussion.

B. The effect of carbon monoxide, azide and antimycin A on DNP-stimulated respiration

Diapausing *Cynthia* pupae were injected with DNP and then exposed to carbon monoxide. The results, summarized in Figure 6, reveal that about half the DNP-stimulated respiration was inhibited by carbon monoxide. Further analysis of the data from this experiment revealed that CO-sensitivity increased slightly as the rate of oxygen consumption increased. Thus pupae with a DNP-stimulated respiration of $90 \text{ mm}^3/\text{gm. live wt./hr.}$ had only 45 per cent of their respiration inhibited by carbon monoxide, whereas pupae with a DNP-stimulated respiration of $125 \text{ mm}^3/\text{gm. live wt./hr.}$ had nearly 70 per cent of their respiration inhibited by carbon monoxide.

The effect of azide on DNP-stimulated respiration of diapausing *Cynthia* pupae is recorded in Figure 7. There was no significant initial inhibition of the respiration when sodium azide alone was injected (see Section 1D), but some of the DNP-stimulated respiration was inhibited by this reagent. Indeed, as Figure 7B shows, more than three-fourths of the DNP-stimulated respiration was inhibited by 5×10^{-4} M sodium azide. However, in group B only half of the pupae receiving injections of DNP and none of the pupae receiving sodium azide survived for more than a week, indicating these high concentrations of antimetabolites were ultimately toxic. Comparable results were obtained with *Cecropia* pupae.

Experiments appraising the antimycin A-sensitivity of DNP-stimulated respiration were conducted on a series of 15 *Cynthia* pupae which received 10^{-4} M DNP and 10^{-6} M antimycin A. About 30 per cent of the DNP-stimulated respiration was inhibited by this concentration of inhibitor. Thus, the respiration of DNP-stimulated pupae is no more sensitive to antimycin A than the respiration of normal pupae.

3. Injury-stimulated respiration

A. The CO-sensitivity of injury-stimulated respiration

As mentioned previously, integumentary injuries to pupae dramatically accelerate respiration for one to three weeks (Schneiderman and Williams, 1953a, 1953b). Moreover, this accelerated respiration is proportional to the extent of injury and seems to be caused in part by diffusible substances released at the site

of injury (Jankowitz, 1955; Schneiderman, 1957). Although the respiration induced by a small incision into *Cecropia* pupae was not inhibited by carbon monoxide, repair of extensive wounds was prevented by this gas (Schneiderman and Williams, 1953b, 1954b), suggesting that the cytochrome oxidase system was functioning in injured pupae.

To investigate this possibility the following experiments were carried out. Four *Cynthia* pupae were given a large injury by removing their pupal legs; two pupae

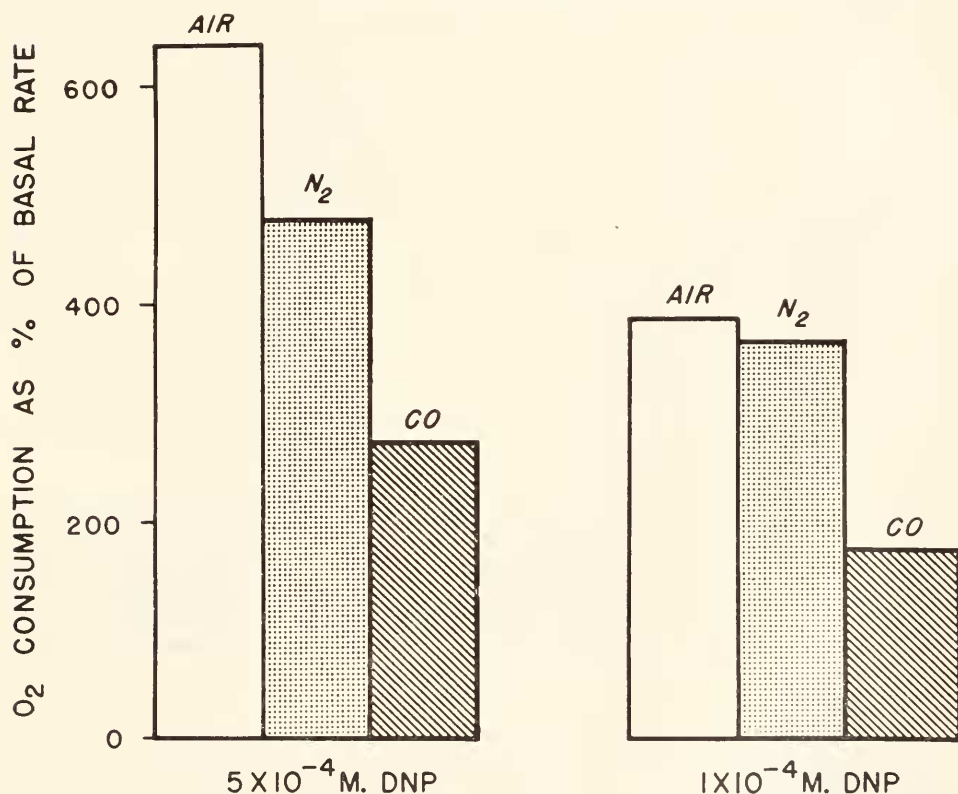


FIGURE 6. The CO-sensitivity of DNP-stimulated respiration. Two groups of five diapausing *Cynthia* pupae were injected with DNP to internal concentrations of $5 \times 10^{-4} M$ and $10^{-4} M$. All the pupae were exposed to 5% O₂ and 95% N₂ and then to 5% O₂ and 95% CO (CO/O₂ = 19:1). The average respiration over a one-hour period is recorded.

were immediately placed in 7 per cent oxygen and nitrogen, and the other two were placed in a corresponding atmosphere of oxygen and carbon monoxide. The pupae were maintained in their respective gas mixtures for one week, and the gas mixtures were renewed thrice daily.

As Figure 8 shows, injured pupae in the nitrogen mixture developed a characteristic injury respiration; on the other hand, those in carbon monoxide mixtures did not. Indeed, five days after injury both of the pupae maintained in carbon monoxide had died. Thus carbon monoxide apparently caused death by preventing

the development of injury respiration. Similar results were obtained with *Prometha* pupae. These results are in general agreement with those of Schneiderman and Williams (1954b), who reported that the repair of injury was CO-sensitive. However, their experiments failed to detect the CO-sensitivity of the respiration associated with repair of injury, presumably because they employed only small injuries. Such CO-sensitivity was demonstrated by Harvey and Shappirio (Harvey, 1956) who pointed out that after very large injuries respiration becomes sensitive to carbon monoxide. This is confirmed in the following experiment summarized in

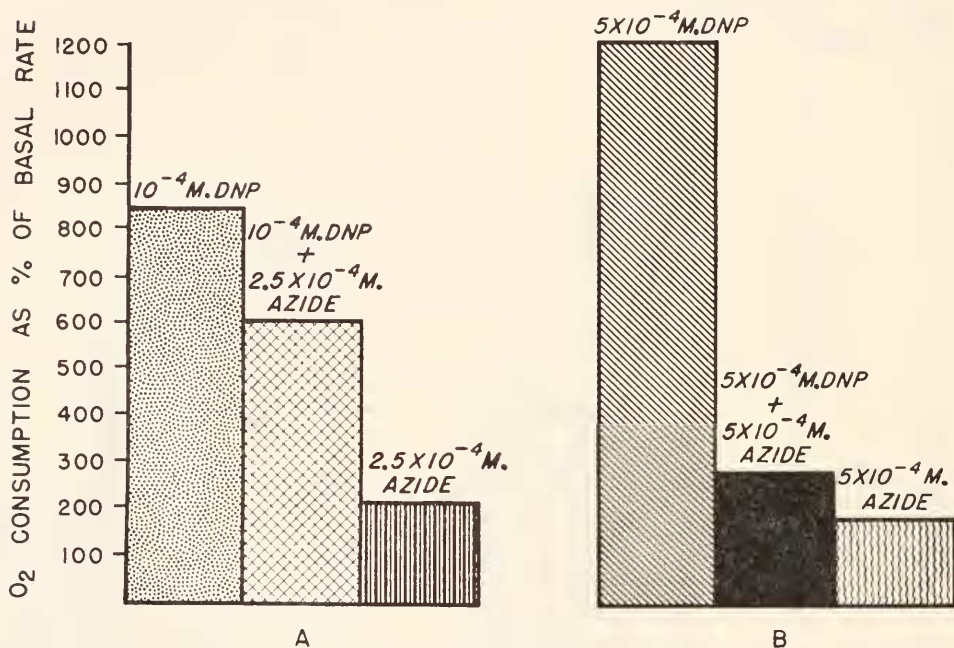


FIGURE 7. The azide-sensitivity of DNP-stimulated respiration. (A) Four diapausing *Cynthia* pupae were injected with 10⁻⁴ M DNP, four with 2.5 M sodium azide, and four with both reagents. The average O₂ consumption over a three-hour period is recorded. (B) Five pupae were injected with 5 × 10⁻⁴ M DNP, five with 5 × 10⁻⁴ M sodium azide and five with both reagents. The average O₂ consumption over a three-hour period is recorded. The average initial oxygen consumption of the pupae in (A) and (B) was 16.8 mm.³/gm. live wt./hr.

Figure 9. Four brainless *Cynthia* pupae were injured by removing the pupal legs and after three days, when they had developed a large injury respiration, the CO-sensitivity of their respiration was determined. About two-thirds of the injury respiration was inhibited by carbon monoxide. Similar results were obtained with *Cecropia* pupae. It can also be seen in Figure 9 (as well as in Figure 6) that the oxygen uptake of pupae respiring at a rapid rate was limited by the low oxygen tension. This contrasts with the respiratory behavior of pupae with low metabolic rates, where 5 per cent oxygen and 95 per cent nitrogen commonly stimulated oxygen consumption (see Section 1C).

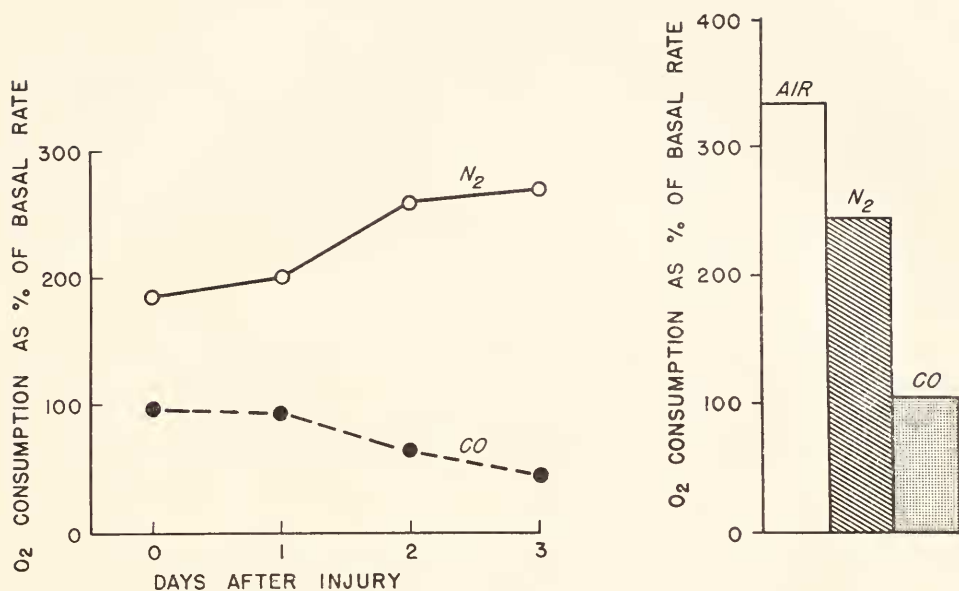


FIGURE 8 (left). The effect of injury and simultaneous exposure to CO on respiration. Two injured *Cynthia* pupae were maintained continuously in 7% O₂ plus N₂ and two were maintained in 7% O₂ plus CO (CO/O₂ = 13:1). The average respiration of each pair over a 3-hour period is recorded as a function of time. The day of injury is denoted as day "0".

FIGURE 9 (right). The CO-sensitivity of injury respiration. The average respiration over a 4-hour period of four brainless *Cynthia* pupae 3 days after injury in air, in 5% O₂ and 95% N₂, and in 5% O₂ and 95% CO (CO/O₂ ratio = 19:1).

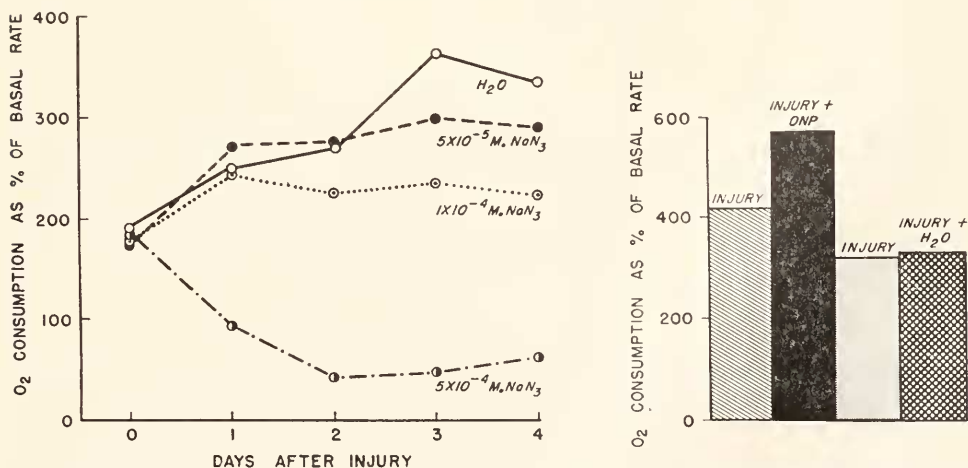


FIGURE 10 (left). The effect of azide injection on the O₂ consumption of four groups of five diapausing *Cynthia* pupae over a five-day period. The day of injection is denoted as day "0".

FIGURE 11 (right). The effect of DNP on injury respiration. The average O₂ consumption over a 3-hour period of two injured diapausing *Cynthia* pupae prior to and after the injection of water, and of two injured pupae prior to and after the injection of 5 × 10⁻⁴ M DNP.

B. *Injury-stimulated respiration in newly pupated Cecropia*

Four *Cecropia* silkworms were injured within one day after pupation by removing a rectangular window of pupal cuticle from their faces. No significant stimulation of respiration was observed. Since injury respiration is characteristic of diapausing pupae, and since the respiration of newly pupated *Cecropia* is much greater than the respiration of pupae firmly in diapause, this result suggests that the production of injury respiration is intimately associated with the extremely low respiration of the diapausing insect (see Discussion).

C. *The azide-sensitivity of injury-stimulated respiration*

The effect of azide on injury respiration was examined by injecting a series of diapausing *Cynthia* pupae with sodium azide at several concentrations. In this experiment the injection itself served as the injury. The average daily respiration of each group of pupae over a five-day period is plotted in Figure 10.

All pupae treated with 5×10^{-4} M sodium azide died within 10 days after injection.

TABLE II
Effect of simultaneous injury and injection of DNP on the respiration of six diapausing Cynthia pupae

Basal respiration, mm. ³ /gm./hr.	Treatment	Max. resp. as % basal rate	Day of max.
25.0	Injury + H ₂ O	258	2
12.0	Injury + H ₂ O	521	2
31.0	Injury + H ₂ O	234	3
26.5	Injury + DNP	449	6
40.5	Injury + DNP	496	6
30.5	Injury + DNP	Died	—

tion, indicating either (a) that this concentration had a simple toxic effect, or (b) that the development of injury-stimulated respiration was inhibited by azide and this caused death, as was the case when injured pupae were exposed continuously to mixtures of carbon monoxide and oxygen (see Section 3A above). In lower concentrations of azide, the inhibition of injury respiration was proportional to concentration.

D. *The effects of DNP on injury-stimulated respiration*

The pupal legs were removed from a group of four *Cynthia* pupae. Three days after wounding, when injury respiration had reached its maximum, two of the pupae received injections of DNP to an internal concentration of 5×10^{-4} M, and the remaining two received injections of water. The data summarized in Figure 11 show that 5×10^{-4} M DNP caused a significant acceleration of maximum injury respiration; however, the increase was proportionately much less than that encountered in DNP-treated diapausing pupae. Comparable results were obtained with *Cecropia* and *Promethea* pupae.

In another experiment, six diapausing pupae were injured by removing their

pupal legs; half these pupae immediately received water injections, while the remainder received injections of DNP to an internal concentration of 5×10^{-4} M. The respiration of these pupae is summarized in Table II. The maximum respiration of injured pupae treated with DNP was reached six days after the injury, while those receiving injections of water displayed maximum respiration two or three days after injury. Thus DNP delayed the development of injury respiration.

DISCUSSION

1. *A new explanation for the insensitivity of pupal respiration to carbon monoxide*

Studies noted in the Introduction have shown that the onset of pupal diapause in giant silkworms is accompanied by a precipitous fall in the rate of oxygen consumption, and that the low respiration of the diapausing pupa is virtually uninhibited by carbon monoxide and cyanide. As judged by its insensitivity to these inhibitors, nearly all of the respiration of the diapausing pupa appeared to proceed *via* pathways independent of cytochrome oxidase. Hence it was suggested that the respiration of the diapausing pupa was mediated by a terminal oxidase other than cytochrome oxidase, possibly a flavoprotein or an autoxidizable cytochrome of the *b* type (Schneiderman and Williams, 1954a, 1954b). This suggestion was taken up by various investigators (Cotty, 1956; Ito, 1955). The present experiments provide an alternative explanation for the CO-insensitivity of pupal respiration; namely, that it is due to a great excess of cytochrome oxidase relative to trace amounts of cytochrome *c* in most of the tissues of the diapausing pupa. This limitation of cytochrome *c* leads to an unsaturation of cytochrome oxidase, and this in turn leads to the insensitivity of pupal respiration to carbon monoxide and azide. Under this view the principal factor underlying the *low respiration* of the diapausing pupa is the *limitation of cytochrome c* in most of the pupal tissues, while the principal factor underlying the *CO- and azide-insensitivity* of pupal respiration is the *excess of cytochrome c oxidase* in most of the pupal tissues. Thus, quantitative changes in the relative amounts of respiratory enzymes after pupation are responsible for both the low over-all respiration of diapause and for CO-insensitivity. In other words, the basic differences between the respiratory enzyme systems of diapausing and non-diapausing insects are *quantitative*, but they lead to *qualitative* differences in the response of the insect to certain inhibitors. Contrary to earlier opinions, cytochrome oxidase appears to be the principal terminal oxidase during diapause as well as during all the other stages of the life history.

2. *Preliminary theoretical considerations*

It can be shown that an excess of cytochrome oxidase may lead to a virtual CO-insensitivity of respiration that is actually mediated by cytochrome oxidase, and in the final section of this discussion a brief theoretical analysis of this assertion is presented. The argument offered is that when cytochrome oxidase is in great excess and thus not saturated, a large fraction of the cytochrome oxidase may be inhibited by carbon monoxide without affecting the rate of electron transfer from cytochrome *c*. Stated in another way the greater the "saturation" of cytochrome oxidase by cytochrome *c*, the greater the CO-sensitivity of respiration; the less the "saturation" of cytochrome oxidase by cytochrome *c*, the less the CO-

sensitivity. This conclusion seems intuitively acceptable and is proven in Section 9 (below). Recognizing this relation between CO-sensitivity and saturation of cytochrome oxidase it is not difficult to interpret the several experimental results.

3. Carbon monoxide experiments

Evidence presented previously has shown that the specific target of carbon monoxide in the insect at all stages is reduced cytochrome *c* oxidase (Schneiderman and Williams, 1954a, 1954b). A principal factor determining the impact of carbon monoxide on cytochrome oxidase is the CO/O₂ ratio: the higher this ratio, the greater the proportion of reduced cytochrome oxidase molecules inhibited. The CO/O₂ ratios employed in the present experiments were usually 16:1 or 19:1 and ambient oxygen tensions were maintained at 6 or 5 per cent. Direct analysis of the composition of the tracheal gas of normal diapausing pupae kept at these oxygen tensions by a precise microgasometric method (Levy and Schneiderman, 1957, 1958) revealed that the actual oxygen tension within the tracheal system, and hence within the insects' tissues, was about 1 per cent lower than ambient, that is, about 5 or 4 per cent. Therefore in the present experiments the actual CO/O₂ ratios within the pupal tissues approached 24:1. Since it has been shown that a CO/O₂ ratio of 16:1 causes a 50 per cent light-reversible inhibition of the cytochrome oxidase activity of homogenates of the thoracic muscles of *Cecropia* moths (Pappenheimer and Schneiderman, unpublished), we may conclude that *the CO/O₂ ratios used in the present experiments were capable of inhibiting no less than 50 and probably as much as 75 per cent of the reduced cytochrome oxidase activity of homogenates of the insect's tissues.* However, as we have already noted in the previous section, the inhibition in a homogenate where cytochrome oxidase is saturated by added cytochrome *c* may be quite different from the inhibition observed in the intact insect where the cytochrome oxidase may not be saturated by cytochrome *c*. Let us now consider what our several experiments tell us about the saturation of cytochrome oxidase in the diapausing pupa.

Perhaps the most crucial result is recorded in Figure 2. As the figure shows, when the oxygen tension is reduced to 2 per cent in a mixture of oxygen and nitrogen, the oxygen consumption of the pupa remains about the same as in air, but the CO-sensitivity of the respiration is enhanced. The simplest interpretation of this result is that cytochrome oxidase is present in excess over some rate-limiting link in the respiratory chain, and only at low oxygen tensions does the cytochrome oxidase-oxygen reaction become the limiting step in the respiratory chain, subject, as a consequence, to inhibition by carbon monoxide.

The reasons for stimulatory effects of carbon monoxide on pupae with low metabolic rates (*cf.* Fig. 1) are not yet clear. Similar stimulatory effects of carbon monoxide have been reported by Bodine and Boell (1934a) for *Melanoplus*, by Klein and Runnström (1940) for unfertilized eggs of the sea urchin, and by others (*cf.* review by Needham, 1942, p. 496). Possibly it does not represent stimulation of respiration but is simply gas uptake due to an actual oxidation of CO by the tissues to CO₂ (*cf.* review of Lilienthal, 1950). Perhaps it is something different altogether, such as an uncoupling action (Thimann *et al.*, 1954). For our present purposes suffice it to say that the phenomenon, although not yet explained, does not affect our interpretation of the basic action of carbon monoxide

on cytochrome oxidase and the argument that cytochrome oxidase is only partially saturated in pupal tissues. Further evidence supporting this argument derives from studies with DNP and azide which are considered in Sections 4 and 5 below.

Significant data revealing the degree of saturation of cytochrome oxidase in the diapausing pupa are also to be found in the observation that CO-sensitivity of pupal respiration increases with increasing basal respiration and is instantly enhanced by DNP, and in the fact that the increased respiration that follows injury or the initiation of adult development is inhibited by carbon monoxide. Moreover, we have found that the increased respiration that follows a prolonged period of anoxia is also sensitive to carbon monoxide. These results, which are summarized in Table III,

TABLE III

Summary of the effects of metabolic inhibitors on the respiration of diapausing pupae in various physiological states and on developing adults

Effect				
Physio- logical condition \ Inhibitor	CO CO : O ₂ = about 20:1	DNP	Azide	Antimycin A
Diapause respiration	Stimulation at low basal rates Slight or no inhibition at modest basal rates. Inhibition increases as basal respiration increases Up to 50% inhibition at low oxygen tensions	5×10^{-4} M stimulates respiration an average of 12-fold and as much as 16-fold Stimulation less at high basal rates	No immediate effect at concentrations up to 5×10^{-4} M	30% inhibition at 10^{-5} M
DNP-stimulated respiration	An average of 50% inhibition	—	30 to 70% inhibition depending on concentration of azide	30% inhibition at 10^{-5} M
Injury-stimulated respiration	No or slight inhibition after small injury; up to 60 per cent inhibition after large injury Exposure immediately after injury prevents development of injury-stimulated respiration	Stimulation by DNP inversely proportional to size of injury-stimulated respiration. After large injuries, about 2-fold stimulations by DNP. Injection of DNP immediately after injury delays development of injury respiration	Inhibition proportional to concentration of azide	—
Developing adult	More than 50% inhibition (Schneiderman and Williams, 1954a)	5×10^{-4} M stimulates respiration about 2-fold	—	—

lead to the conclusion that *the fraction of respiration sensitive to carbon monoxide is a function of the rate of oxygen consumption of the silkworm at all stages*. This implies that virtually any process which increases the rate of pupal respiration increases the saturation of cytochrome oxidase and that, in the pupa, cytochrome oxidase is in great excess and hence very unsaturated.

Recognizing the importance of low over-all respiratory rate as a factor in CO-insensitivity, it is worthwhile considering certain diapausing insects whose respiration is not resistant to carbon monoxide or cyanide. Two species whose respiration continues to be inhibited by carbon monoxide or cyanide during diapause are prepupae of the larch sawfly, *Pristophora* (McDonald and Brown, 1952), and larvae

of the horse bot fly, *Gastrophilus* (Levenbook, 1951). It is of considerable significance that the respiration of these insects at 25° C. is many times greater than the respiration of diapausing silkworm pupae. Thus the respiratory rate of *Pristophora* is about 165 mm.³/gm. live wt./hr., while that of *Gastrophilus* is more than 100 mm.³/gm. live wt./hr.³ This compares with a respiratory rate for diapausing silkworm pupae of 8 to 20 mm.³/gm. live wt./hr. Furthermore, in diapausing silkworm pupae only the skeletal muscles, of which there are few, have a saturated cytochrome *c* oxidase, and these account for only a small fraction of the insect's total respiration. In diapausing species with high respiratory rates like *Pristophora* and *Gastrophilus* it appears likely that (1) they have more muscular tissue and this accounts for a larger fraction of their total respiration than do the muscles of diapausing pupae, and (2) some of their non-muscular tissues may have a saturated cytochrome oxidase. These factors could easily account for their sensitivity to carbon monoxide.

4. The significance of DNP-stimulated respiration

The experiments with DNP demonstrate that in diapausing pupae cytochrome oxidase is not fully saturated. As is well known, DNP increases the turnover of the respiratory carriers, presumably because it is able to uncouple phosphorylation from electron transfer, and so increases the demand for oxygen (Chance and Williams, 1956). The data in Section 2 reveal a striking 12- to 16-fold acceleration of pupal oxygen consumption by 5×10^{-4} M DNP. This may be one of the largest DNP stimulations ever recorded. It contrasts with the finding of Bodine and Boell (1938) that the respiration of diapausing *Melanoplus* eggs was accelerated a maximum of only 3.5 times by 3×10^{-5} M DNP, while further increase in concentration produced a submaximal response. De Meio and Barron (1934) and Maroney *et al.* (1957) have reported DNP stimulations in various invertebrate tissues of only about two-fold. Aside from the magnitude of DNP-stimulated respiration (which by itself suggests unsaturation of cytochrome oxidase), the CO-sensitivity of DNP-stimulated respiration is of special interest. It indicates that DNP accelerates the turnover of several carriers of the respiratory chain but has a lesser effect on the turnover of cytochrome oxidase. This conclusion arises from the fact that CO-sensitivity is a function of the saturation of cytochrome oxidase. The CO-sensitivity of DNP-stimulated respiration tells us that DNP increases the saturation of cytochrome oxidase. Thence it follows that DNP not only accelerates over-all respiratory rate but alters the quantitative relationship between cytochrome oxidase and the intermediate carriers in the respiratory chain.

One of these accelerated carriers is almost certainly cytochrome *c*, which may be the most important rate-limiting carrier in the respiratory chain, a point we shall consider further in Section 6 (below). Dinitrophenol appears to increase in some way the effective turnover of this enzyme and increases thereby the saturation of cytochrome oxidase. It is significant that both the absolute magnitude and the CO-sensitivity of DNP-stimulated respiration were lower for pupae with low basal respiration. Thus in the present experiments, although pupae with low basal metabolic rates were proportionately more stimulated by DNP than pupae with high basal metabolic rates, the latter developed a greater over-all respiration under

³ This last value was calculated from values obtained at 37° C. by assuming a Q_{10} of about 2.5.

the influence of DNP. The data also reveal that CO-sensitivity reached a maximum of about 70 per cent when DNP-stimulated respiration reached its maximum. We interpret these findings to mean that pupae with low basal respiration have less cytochrome *c* available to be turned over and, as a result, these pupae are not capable, even under the influence of high concentrations of DNP, of completely saturating their cytochrome *c* oxidase and thereby achieving maximum CO-sensitivity. These DNP studies provide support for the argument that the low over-all respiration of diapause is due to a low concentration of some respiratory component, probably cytochrome *c*, whereas the CO-insensitivity is the result of the relatively high concentration of cytochrome *c* oxidase.

In our experience the respiration of developing adults is accelerated by DNP to a much lesser extent than that of diapausing pupae, usually about two-fold. This fact suggests that in the developing adult, as contrasted with the diapausing pupa, cytochrome oxidase is virtually saturated. Also, although development may be delayed, developing adults survive concentrations of DNP which are toxic to diapausing pupae, possibly because their higher metabolic rate enables them to metabolize the DNP (cf. Cross *et al.*, 1949).

5. *The significance of azide-insensitive respiration*

In these insects it seems safe to identify cytochrome oxidase as the main target of azide (Horecker and Stannard, 1948; Stannard and Horecker, 1948). The experiments summarized in Section 1D disclosed that azide had no immediate effect on diapause respiration at concentrations as high as 5×10^{-4} M. This result supports the conclusion drawn above, that cytochrome oxidase does not limit pupal respiration. On the other hand, the sensitivity to azide of DNP-stimulated respiration was quite striking. This is consistent with the argument that, under the influence of DNP, cytochrome oxidase becomes more saturated.

6. *The limiting link in the pupal respiratory chain*

The present experiments provide only one clue to the identity of the limiting link in the pupal respiratory chain. This is the fact that antimycin A—a potent inhibitor at the concentrations we employed of the DPNH-cytochrome *c* reductase system—had only a minor effect on normal pupal respiration and DNP-stimulated respiration. This inhibitor is said to have as its specific target the Slater factor which mediates the transfer of electrons from flavoprotein to cytochrome *c* (Potter and Reif, 1952; Reif and Potter, 1953; Chance and Williams, 1956). The insensitivity of pupal respiration to this reagent suggests that the limiting link in the pupal respiratory chain lies between the Slater factor and cytochrome oxidase, *e.g.*, cytochrome *c*. Recent studies of Shappirio and Williams (1957a, 1957b) indicate that the limiting link is very likely cytochrome *c*, for with very sensitive spectroscopic techniques they were unable to detect this enzyme in most pupal tissues although cytochrome oxidase was easily demonstrated. They also showed that in homogenates of pupal tissues, cytochrome *c* is a rate-limiting link in the oxidation of DPNH. Hence it seems safe to identify limiting concentrations of cytochrome *c* as a principal cause of the unsaturation of cytochrome oxidase in pupal tissues.

7. Injury-stimulated respiration

The increased sensitivity to carbon monoxide and azide shown by pupae supporting an injury respiration (Sections 3A and 3C) indicate an increased saturation of cytochrome oxidase after injury. The observation (Section 3D) that 5×10^{-4} M DNP failed to accelerate injury respiration to the same degree as diapause respiration supports the conclusion that cytochrome oxidase is virtually saturated when injury respiration is at its maximum. What brings about this increased saturation of cytochrome oxidase is not known with certainty but the present experiments suggest that it is caused by a gradual synthesis of cytochrome *c* which is provoked by injury. Recall that injured pupae treated with DNP were delayed in developing maximum injury respiration when compared with injured pupae receiving water injections. This suggests that integumentary injury initiates some process which requires a supply of phosphate bond energy which was uncoupled by DNP. The gradual development of maximum injury respiration over a three-day period suggests further that this energy-demanding process involves, in part, the synthesis of one or more of the respiratory chain components and does not simply reflect increased turnover of pre-existing enzymes. We interpret the increased CO-sensitivity of injury-stimulated respiration to indicate that more cytochrome *c* is being synthesized than cytochrome oxidase. That augmented protein synthesis does in fact follow injury has been demonstrated by Telfer and Williams (1955), who showed that the incorporation of C^{14} -labelled glycine into the pupal proteins was stimulated by injury to about the same extent as respiration.

It is not without interest that the synthesis of these respiratory components appears to be obligatory. Indeed, the data in Section 3A suggest that when synthesis is prevented by prolonged exposure to carbon monoxide, the pupae fail to develop an injury respiration and die. This obligatory synthesis of new respiratory components may be imposed upon diapausing pupae because their capacity for wound repair is restricted by their low metabolic rate. Apparently this repair process is able to compete with the "maintenance" processes of the diapausing pupa, thereby causing death when total energy production is reduced by carbon monoxide. In this connection, it is noteworthy that newly molted pupae, whose respiratory rate is considerably larger than that of pupae firmly in diapause, fail to show an injury respiration. This reflects their capacity to underwrite the energy requirements of injury without augmenting the respiratory chain. This capacity is also present in developing *Cecropia* adults and we have also shown it in all stages of non-diapausing species such as the bee-moth *Galleria mellonella*.

8. Conclusions

The several lines of evidence considered in the preceding sections persuade us that earlier conceptions of the respiratory enzyme system of diapausing silkworms need re-evaluation. The basic differences between the respiratory enzyme chains of the diapausing pupa and the non-diapausing stages appear to be *quantitative* differences and not *qualitative* differences as was suggested earlier (Williams, 1951; Schneiderman and Williams, 1954a, 1954b). The CO-insensitivity of pupal respiration does not stem from the activity of a CO-insensitive terminal oxidase, but results from a great excess of cytochrome oxidase relative to other components of the

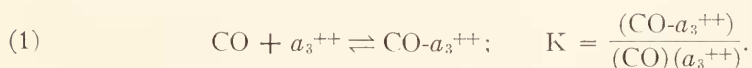
respiratory chain. None of our findings supports the renewed suggestions of Wojtczak (1955) and Ito (1955) that tyrosinase functions as a terminal oxidase in insects. Indeed, in view of the failure of potent inhibitors of tyrosinase like phenylthiourea to inhibit respiration (Schneiderman and Williams, 1954a) and the light-reversibility of the carbon monoxide inhibition of silkworm growth (Schneiderman and Williams, 1954b) and respiration (Pappenheimer and Schneiderman, unpublished) this is not likely. The present data, coupled with the recent spectroscopic findings of Shappirio and Williams (1957a, 1957b) and with the studies of Harvey and Williams (1958a, 1958b) on the pupal heart, indicate that cytochrome oxidase is the terminal oxidase during pupal diapause and cytochrome *c* is the limiting component in the pupal respiratory chain.

In this perspective, the increased respiration following integumentary injury and initiation of adult development reflects an increase in cytochrome *c* content which occurs at a faster rate than any increase in cytochrome oxidase. Possibly the increase in cytochrome *c* reflects its adaptive synthesis in response to changes in the energy requirements of the tissues. These changes were induced on the one hand by injury and on the other by the prothoracic gland hormone which initiated adult development. Such an adaptive synthesis of cytochrome *c* has been suggested in the case of regenerating rat liver by Drabkin (1955). However, while the data support the view that cytochrome *c* is the limiting link in the pupal respiratory chain, they do not rule out the possibility that other factors, such as phosphate acceptors, may exert short-term effects on pupal respiration.

In conclusion, it is worth recalling that many animals other than diapausing pupae of the silkworm have a low respiration that is insensitive to carbon monoxide. Moreover, in many of these, such as diapausing eggs of grasshoppers and silkworms and unfertilized eggs of sea urchins, cytochrome oxidase is clearly present. The usual explanation for CO-insensitivity has been that respiration proceeded along tracks alternative to the cytochrome oxidase system (*cf.* Needham, 1942, p. 567). It is noteworthy, however, that in interpreting some of the very first experiments which showed this CO-insensitivity, Runnström (1930) suggested that cytochrome oxidase was not saturated with its substrate and this was the reason for CO-insensitivity in the sea urchin egg. In retrospect, it seems likely that this idea was sound and that the CO-insensitivity of the respiration of many systems is probably the result of an excess of cytochrome oxidase relative to some other component of the respiratory chain.

9. *Final theoretical considerations of carbon monoxide-insensitive respiration*

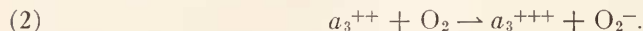
The basic premise underlying the arguments offered in the earlier sections of this discussion is that an excess of cytochrome oxidase can lead to a virtual carbon monoxide-insensitivity of a cytochrome oxidase-mediated respiratory chain. This is shown as follows. It is well known that carbon monoxide combines only with the reduced form of cytochrome oxidase (also called a_3):



Equation (1) is the simple chemical equilibrium with a characteristic equilibrium constant that describes the interaction of reduced cytochrome oxidase (a_3^{++}) with

carbon monoxide. This equation tells us that at a given concentration of carbon monoxide the amount of CO-a_3^{++} complex formed is determined solely by the *steady-state concentration* of reduced cytochrome oxidase.

Now the *steady-state concentration* of reduced (and oxidized) cytochrome oxidase is determined by the rate of electron transfer to cytochrome oxidase, and this, of course, is measured by the rate of oxygen consumption.



Equation (2) describes this *steady-state* between reduced and oxidized cytochrome oxidase. *It is important to note that equation (2) does not describe a simple chemical equilibrium but a steady-state* where the "apparent equilibrium constant" depends on the rate of electron transfer through the respiratory chain. Thus, if the rate of electron transfer to a_3^{+++} from the previous component in the chain is very slow, most of the cytochrome oxidase will be in the oxidized state and the ratio of a_3^{++} to a_3^{+++} will be small. Since the rate of electron transfer is measured by the rate of oxygen consumption, the "apparent equilibrium constant" for equation (2) will vary with the rate of oxygen consumption. This fact, incidentally, rules out the use of the usual Warburg formulation to describe quantitatively the effects of carbon monoxide on respiration, namely

$$(3) \quad \frac{N}{(1-N)} \cdot \frac{\text{CO}}{\text{O}_2} = K,$$

where "N" is the fraction of respiration not inhibited by carbon monoxide (Warburg, 1927). For, this formulation assumes that all the oxidase is in the reduced state, and hence that "the observed respiration is proportional to the amount of enzyme not combined with carbon monoxide" (Warburg, 1949, p. 78). Indeed, Warburg points out that in view of this assumption it is remarkable that there are cells for which his equation applies (p. 79).

When carbon monoxide is used as an inhibitor of cytochrome oxidase, the degree of inhibition of respiration depends upon the new steady-state reached by the system, in which both oxygen and carbon monoxide compete for reduced cytochrome oxidase. In this steady-state, some of the cytochrome oxidase is in the oxidized state, some is reduced and complexed with carbon monoxide, and the remainder is reduced and transferring electrons to molecular oxygen, *i.e.*, playing a role in respiration. The effect of carbon monoxide on respiration depends on the degree to which carbon monoxide decreases the concentration of reduced cytochrome oxidase that is transferring electrons to molecular oxygen. Since a_3^{++} must satisfy the equilibrium conditions of equation (1) and the steady-state conditions of equation (2), it becomes apparent that the amount of a_3^{+++} plays a major role in determining how much a_3^{++} remains to function in respiration. We thus see that the effect of carbon monoxide on respiration depends on the *fraction* of the total cytochrome oxidase in the reduced state. In other words, the effect of carbon monoxide on respiration depends upon the ratio of the actual rate of uninhibited respiration (as measured by the concentration of reduced cytochrome oxidase) to the maximum potential rate of respiration when virtually all the oxidase is kept in the reduced state (as measured

by the total concentration of cytochrome oxidase). This ratio, $\frac{a_3^{++}}{a_3^{++} + a_3^{+++}}$,

the fraction of the total oxidase in the reduced state, is what we ordinarily refer to as the "saturation" of cytochrome oxidase. When the saturation of cytochrome oxidase is high, the carbon monoxide sensitivity is high, and when the saturation is extremely low, the effect of carbon monoxide on respiration is insignificant. This can easily be seen when we consider two extreme cases, bearing in mind equations (1) and (2).

Let us examine a system in which the initial steady-state concentrations of a_4^{++} and a_3^{+++} are about equal (*i.e.*, a high saturation). In such a system, with a 20:1 CO/O₂ ratio an appreciable amount of CO- a_3^{++} can form. When the new steady-state is established in the presence of carbon monoxide, the ratio of the concentrations of a_3^{++} to a_3^{+++} is the same as before. However, the absolute concentration of both these components has been reduced considerably since a large part of the cytochrome oxidase is complexed with the carbon monoxide. As far as respiration is concerned, the significant reduction in a_3^{++} leads to a significant inhibition of respiration by carbon monoxide.

By contrast, consider a system in which the initial steady-state concentration of a_3^{+++} is much greater than the concentration of a_3^{++} . The presence of a CO/O₂ ratio of 20:1 will lead to the formation of only a small concentration of CO- a_3^{++} because of the low concentration of a_3^{++} . Indeed, when the difference between the concentrations of a_3^{+++} and a_3^{++} is very great (*i.e.*, a very low saturation), the total pool of cytochrome oxidase will not be significantly affected by carbon monoxide. As a result, the steady-state concentration of a_3^{++} will not be significantly diminished by the presence of carbon monoxide. Thus the CO-sensitivity of such a system is small.

From the above analysis we learn that an excess of cytochrome oxidase relative to other components of the respiratory chain will lead to CO-insensitivity of respiration. The same conclusion was reached independently by Harvey and Williams (1958b) using a different system and method of analysis.

One further theoretical consideration is crucial to the explanation offered above for CO-insensitivity. If the inhibition of cytochrome oxidase by carbon monoxide is a function of the *total* cytochrome oxidase present, then it must be possible for the transfer of electrons from the carrier part of the respiratory chain to proceed independently of a sterically specific arrangement of the chain components. In their review, Chance and Williams (1956) have discussed this possibility. They concluded that it was highly improbable that the chain components were fixed in position, and they presented two alternatives. Either the chain components were free to act by random collisions according to a modified law of mass action; or, they were fixed in such a manner that the prosthetic groups were free to rotate on an axis and be brought into apposition with adjacent chain components. In either case, electron transfer could proceed across chain components that were not immediately adjacent to one another. *Therefore, it seems possible for the carriers of the respiratory chain of the diapausing pupa to transfer electrons to a "pool" of cytochrome c oxidase. This pool of cytochrome oxidase can manage all of the oxidations, even in the presence of inhibitors, as long as there is sufficient uninhibited enzyme present to meet the needs of electron transfer.* In short, it appears possible for an excess of cytochrome oxidase in tissues to account for the CO- and azide-insensitivity of respiration and of various physiological functions such as heart-beat.

The arguments presented in the previous sections persuade us that this is the situation in most of the tissues of diapausing silkworm pupae.

The experiments reported in Section 1A were performed in collaboration with Dr. Roger D. Smith. We gratefully acknowledge the helpful criticisms of Dr. David P. Hackett, Dr. Conrad S. Yocum, Dr. Carroll M. Williams, and Dr. Howard M. Lenhoff.

SUMMARY

1. To characterize the respiratory enzyme chain that functions during diapause, the respiration of diapausing pupae of the *Cecropia*, *Cynthia*, *Promethea* and *Polyphemus* silkworms was measured in the presence of specific mixtures of oxygen, nitrogen and carbon monoxide, after injection of various metabolic inhibitors and after injury.

2. Pupal respiration is at best only slightly inhibited by carbon monoxide and is often stimulated. Whatever CO-sensitivity there is occurs only in pupae with high basal metabolic rates. Moreover, when respiration is accelerated by injecting dinitrophenol (DNP), or by injury, this evokes an enhanced sensitivity to carbon monoxide. Indeed, it appears that the fraction of respiration sensitive to carbon monoxide is a function of the rate of oxygen consumption of the silkworm at all stages.

3. Reducing external oxygen tension to 2% fails to inhibit oxygen consumption, but increases markedly the CO-sensitivity of pupal respiration. Thus low oxygen tensions seem to unmask CO-sensitivity.

4. Pupal respiration is insensitive to azide concentrations as high as $5 \times 10^{-4} M$. However, the azide-sensitivity, like the CO-sensitivity, increases markedly when pupal respiration is stimulated by DNP or injury.

5. Antimycin A at a concentration of $10^{-6} M$ inhibits less than one-third of normal pupal respiration or DNP-stimulated respiration. Compared to other organisms diapausing pupae are resistant to this inhibitor of the cytochrome *c* reductase system.

6. Dinitrophenol at a concentration of $5 \times 10^{-4} M$ stimulates pupal respiration an average of 12-fold and as much as 16-fold. These are among the largest DNP-stimulations ever recorded. Although pupae with high basal metabolic rates are less stimulated proportionately by DNP than are pupae with low basal metabolic rates, they develop a greater over-all respiration under the influence of DNP.

7. Dinitrophenol-stimulated respiration is inhibited by carbon monoxide. The higher the DNP-stimulated respiration, the greater the inhibition by carbon monoxide. From this and other evidence it appears very likely that DNP accelerates the turnover of one or several components of the respiratory chain while having a lesser effect on cytochrome oxidase.

8. Dinitrophenol delays the appearance of injury-stimulated respiration, suggesting that the development of this increased respiration requires phosphate bond energy. Furthermore, exposure to carbon monoxide causes the death of injured pupae indicating that injury respiration is obligatory and involves the synthesis of new respiratory components.

9. Newly molted pupae not yet firmly in diapause do not respond to wounding with an injury respiration and their respiration is sensitive to carbon monoxide. These findings are correlated with their high respiratory rate.

10. The modes of action of the several inhibitors within diapausing, injured, and developing insects are considered in detail and a new explanation is proposed to account for the CO-, azide-, and cyanide-insensitivity of pupal respiration.

11. It is concluded that the insensitivity of diapausing pupae to inhibitors of cytochrome oxidase results from an excess of this enzyme over its functional requirements in the pupal respiratory chain. This concept is examined in detail and found to be theoretically sound. Evidence is presented that the limiting link in the respiratory chain is cytochrome *c*. Thus, contrary to earlier conceptions, it appears that cytochrome oxidase is the principal terminal oxidase during diapause as well as during all the other stages of the life history, and that the CO-insensitivity of pupal respiration stems from a great excess of cytochrome oxidase relative to cytochrome *c*.

12. The increased CO- and azide-sensitivity of pupal respiration after injection of DNP or injury results from an increase in the saturation of cytochrome oxidase provoked on the one hand by an increase in the turnover rate of cytochrome *c*, and on the other by the synthesis of cytochrome *c*.

13. It is suggested that the CO-insensitivity of the respiration of other organisms may be the result of an excess of cytochrome oxidase relative to some other components of the respiratory chain.

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